

Supplementary Information for:

Nucleotidyl transferase assisted labeling of DNA with different click chemistries

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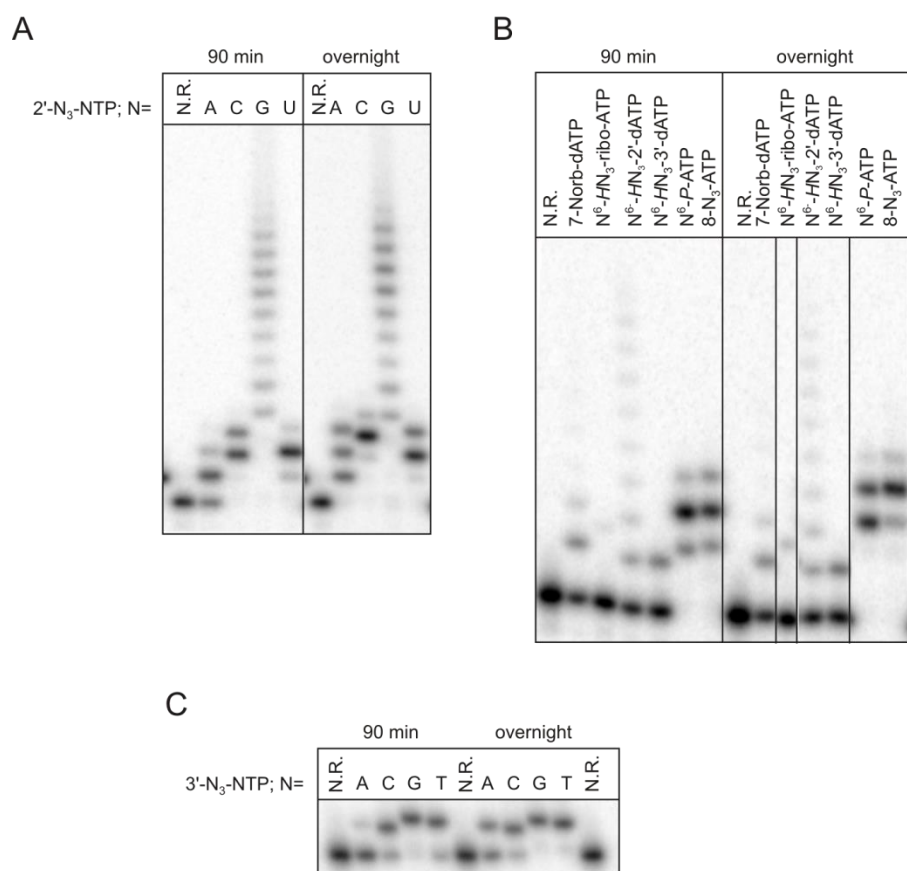
Supplementary Text S1. Detailed comparison of tailing results for non-modified and modified nucleotides.

For most modified 2'-dNTPs we observed shorter and more heterogeneous tail lengths (< 50 nt for 5-*O*-dC/UTP and 5-Norb-dCTP; otherwise < 10 nt; Figures 2B,C, 3A) than for unmodified 2'-dNTPs (> 50 nt; except for 2'-dGTP; Figure 2A). Only the incorporation of 5-*E*-dUTP, in which the small ethynyl-group ($-C\equiv C-H$, see Figure 1C) is directly attached to the base, was comparable to the structurally similar dTTP (Figure 2B). Quantitative turnover of DNA substrate was achieved for most of the pyrimidine-analogs (Figure 2B), but not for the dATP-analogs (7-Norb-dATP, N^6 - HN_3 -dATP; Figure 2C) and 5-Norb-dUTP. Surprisingly, upon a ten and sometimes even 100-fold reduction of nucleotide concentration (Figure 3A), turnover and tail-length increased for several dUTP- and dATP analogs (5-*PEGN*₃-dUTP, 5-DIBAC-dUTP, 5-Norb-dUTP, 7-Norb-dATP, N^6 - HN_3 -dATP), but not for the non-modified ones. We thus conclude that those modified nucleotides exert substrate inhibition, contributing to lower turnover and tail-length. An additional effect has been described previously (39) for ribo-nucleotides: DNA-substrates containing a certain number of these nucleotides are poor substrates, thus limiting the tail-length achieved (see also Figure 2E; tail lengths for ribo-nucleotides always < 10 nt). This may also be the case for specific modified nucleotides and explain shortened tail-lengths. For modified ribo-NTPs incorporation patterns usually resembled those of the respective unmodified ribo-NTPs (Figure 2C,D) but, except for 5-*E*-UTP, tail lengths were somewhat reduced. Here, a reduction of nucleotide concentration generally increased tail-lengths slightly for base-modified (Figure 3B) but not unmodified ribo-NTPs (Figure 3B). In contrast to ribo-NTPs containing modifications attached directly or via short linkers, N^6 - HN_3 -ATP, in which the modification was attached via a longer hexyl-linker, was incorporated with extremely low efficiency [< 2% after 90 min (Figure 2C), or < 5% overnight (Supplementary Figure S1B)]. The turnover increased only marginally upon reduction of nucleotide concentration (to < 10%; Figure 3B). In this study, N^6 - HN_3 -ATP was indeed the nucleotide that exhibited lowest incorporation. Interestingly, its 3'-deoxy-analog (Figure 2C) showed higher incorporation efficiencies at standard conditions. In contrast to the other two analogs, turnover increased to ~100% for N^6 - HN_3 -3'-dATP at 100 μ M and 10 μ M nucleotide concentration (Figure 3B), making it a particularly interesting nucleotide for specific single 3'-terminal modification.

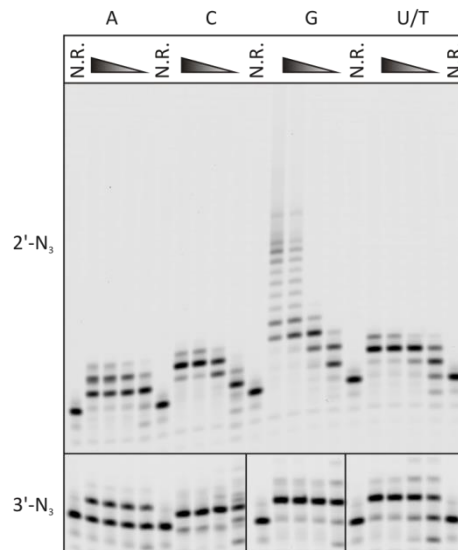
Sugar-modified nucleotides (Figure 2E,F) generally showed similar properties as the respective ribo- (for 2'-N₃) or ddNTPs (for 3'-N₃), with respect to incorporation patterns. Substrate preferences (2'-N₃-G>C≈U>A/3'-N₃-ddG>C≈T>A) were similar to non-modified ribo-nucleotides (G>C>U≈A) but incorporation efficiencies were visibly lower for the sugar-modified nucleotides (except for G). Tail-lengths were reduced for 2'-N₃-A/C/UTP, and for 2'-N₃-ATP, quantitative turnover was achieved only after incubation overnight (Supplementary Figure S1A). After 90 minutes near-quantitative incorporation was achieved for 3'-N₃-ddGTP, but only ~ 70% were observed for 3'-N₃-ddCTP and -TTP (Figure 2A), and low incorporation for 3'-N₃-ddATP [~50% reached only after incubation overnight (Supplementary Figure S1C)].

Supplementary Text S2. Comparison of TdT-catalyzed DNA tailing and PAP-catalyzed RNA tailing – achievability of single nucleotide incorporation.

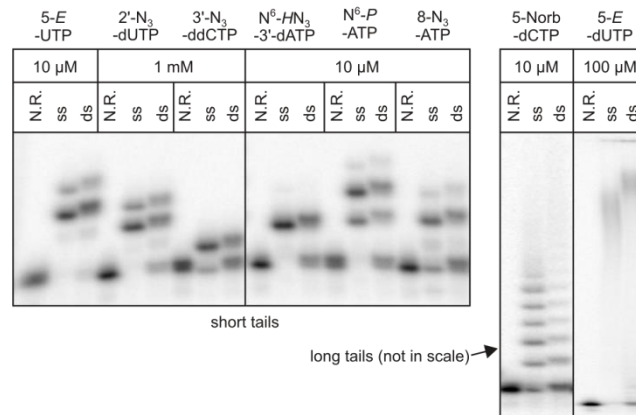
Compared to PAP-catalyzed RNA tailing reactions (9), the TdT-catalyzed DNA tailing reaction depends more strongly on the amount of DNA used, and can often be influenced dramatically by varying the NTP concentration, as well. Therefore, the optimal conditions for the incorporation of a modified nucleotide depend on the experimental requirements and should be adjusted according to the findings mentioned above. Unlike for PAP-catalyzed RNA tailing, TdT-catalyzed DNA tailing could not be manipulated to achieve a very strong preference for single nucleotide incorporation, even by kinetic control (data not shown), presumably because the first and second nucleotide are incorporated at comparable velocity and TdT only seems to discriminate against tailing products after higher numbers of modified nucleotides have been incorporated. Only for few of the modified NTPs (e.g., 5-*E*-UTP, N⁶-*P*-ATP, 8-N₃-ATP, 5-DIBAC-dUTP, and 5-*PEG*N₃-dUTP) reasonable amounts of single-tailed DNA were obtained by using equal amounts of DNA and the respective NTP. However, even in those cases, the products consisted of a mixture of single-, double- and non-tailed DNA. 100% single nucleotide incorporation was therefore only achieved for modified nucleotides with blocked 3'-position (3'-N₃-NTPs or N⁶-HN₃-3'-dATP).



Supplementary Figure S1. Comparison of TdT reaction for 90 min and overnight for selected nucleotides under standard conditions (1 μ M DNA, 1 mM NTP). **(A)** 2'-N₃-NTPs. **(B)** Adenosine-analogs. **(C)** 3'-N₃-NTPs. 15% sequencing PAGE. Radioactive scan is shown. N.R.: not reacted.

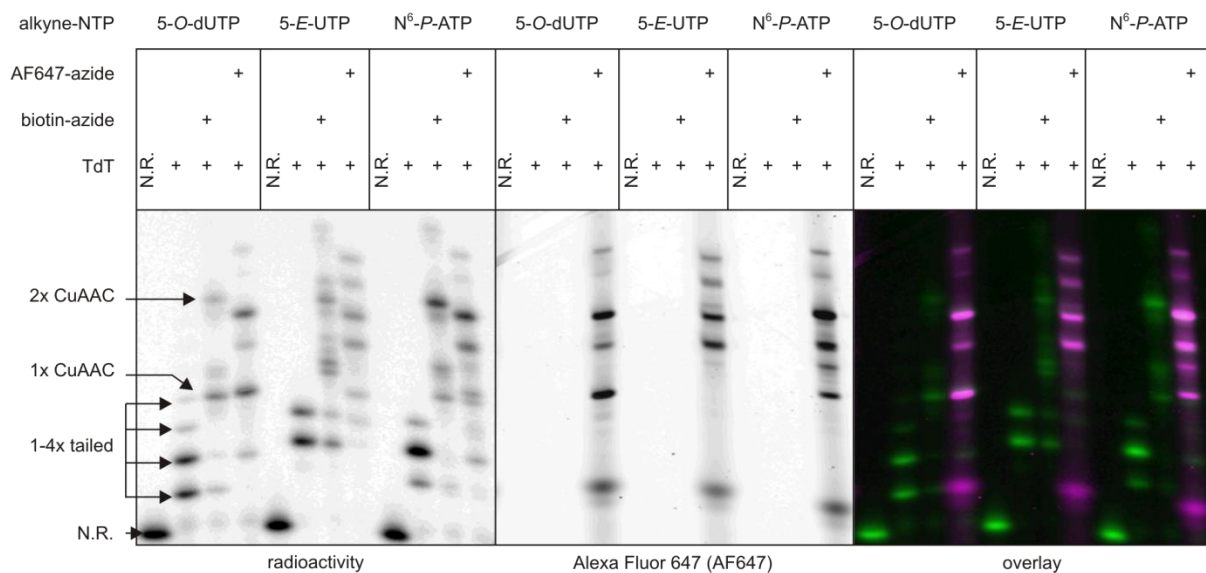


Supplementary Figure S2. TdT reaction (90 min) with sugar-modified nucleotides at different concentrations and DNA4 (1 μ M). 2'-N₃-NTPs (upper panel) or 3'-N₃-ddNTPs (lower panel). N.R.: not reacted. NTP concentration was varied between 1 mM and 1 μ M. Analysis by 15% sequencing PAGE. Fluorescence scan (6-FAM) is shown. Lines indicate where different parts of the same or different gels have been combined for better comparison.

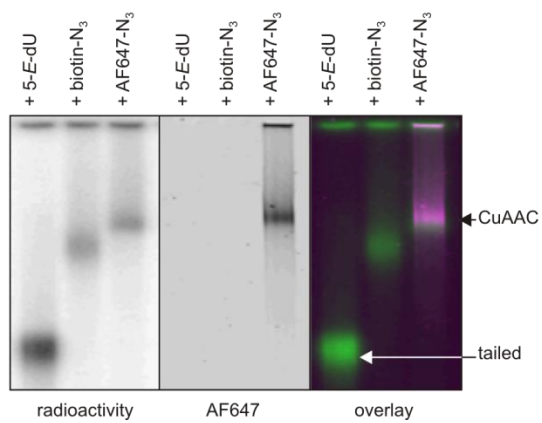


Supplementary Figure S3. TdT tailing (90 min) of ssDNA and dsDNA with different modified nucleotides (concentration as given). ssDNA: DNA2, 10 μ M, dsDNA: DNA2 + DNA2', 0.5 μ M each strand. 15% seq PAGE analysis. Lines indicate where different parts of the same gel were combined for comparison. Phosphor imaging of radioactivity is shown. N.R.: no reaction control. Lines indicate where different parts of the same gel have been combined for better comparison.

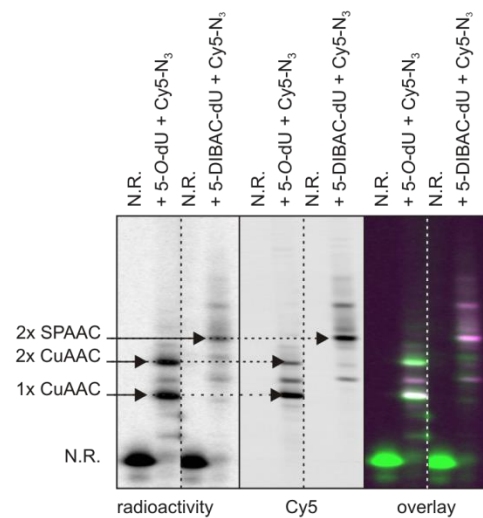
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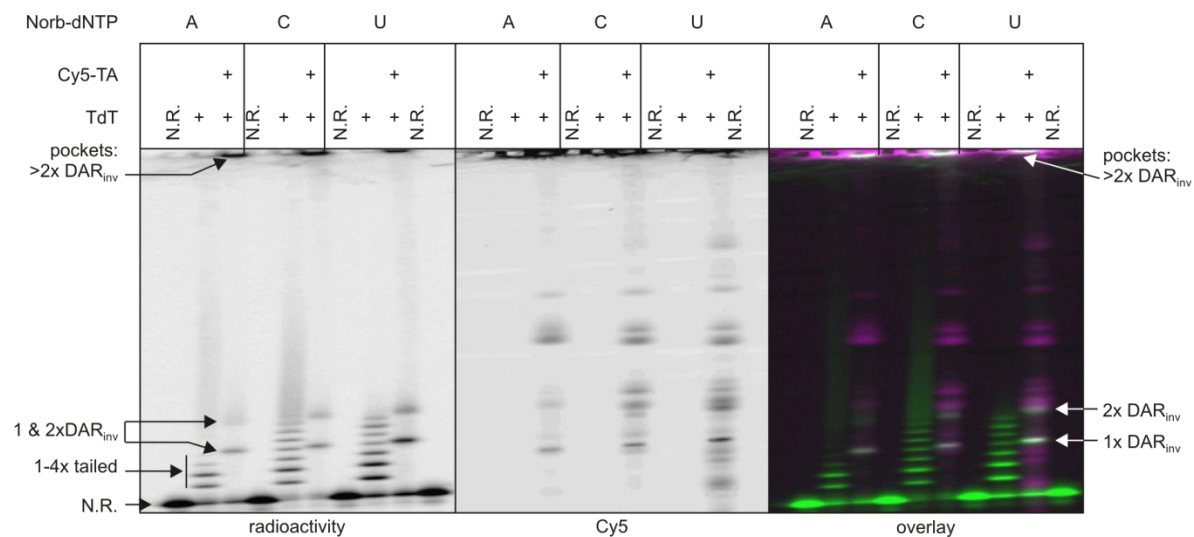
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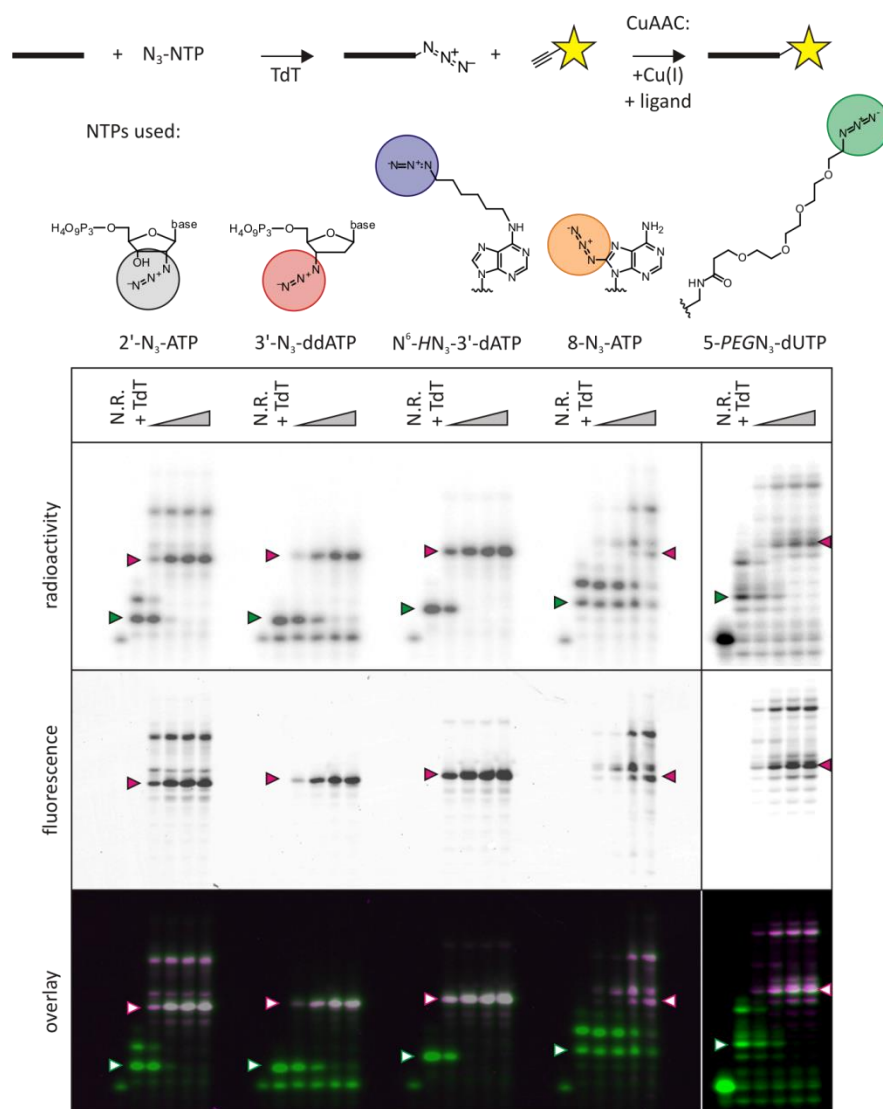
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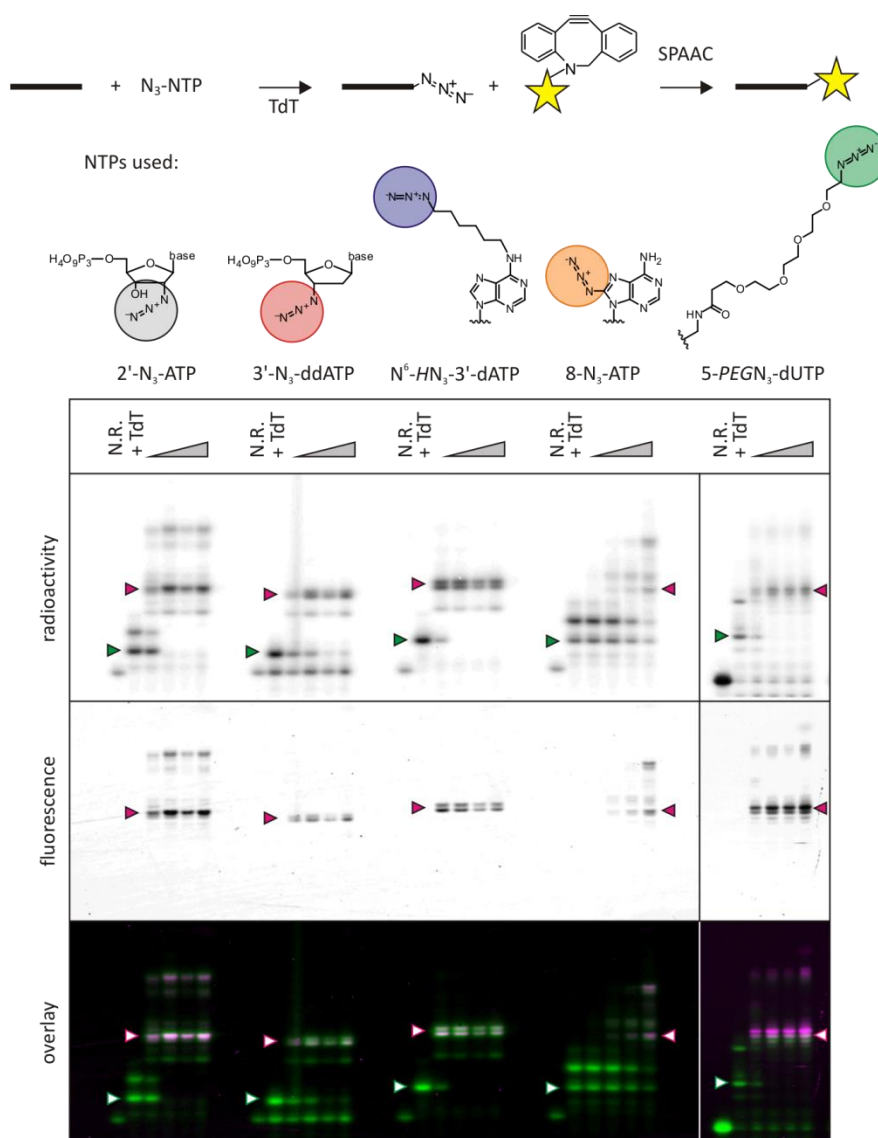
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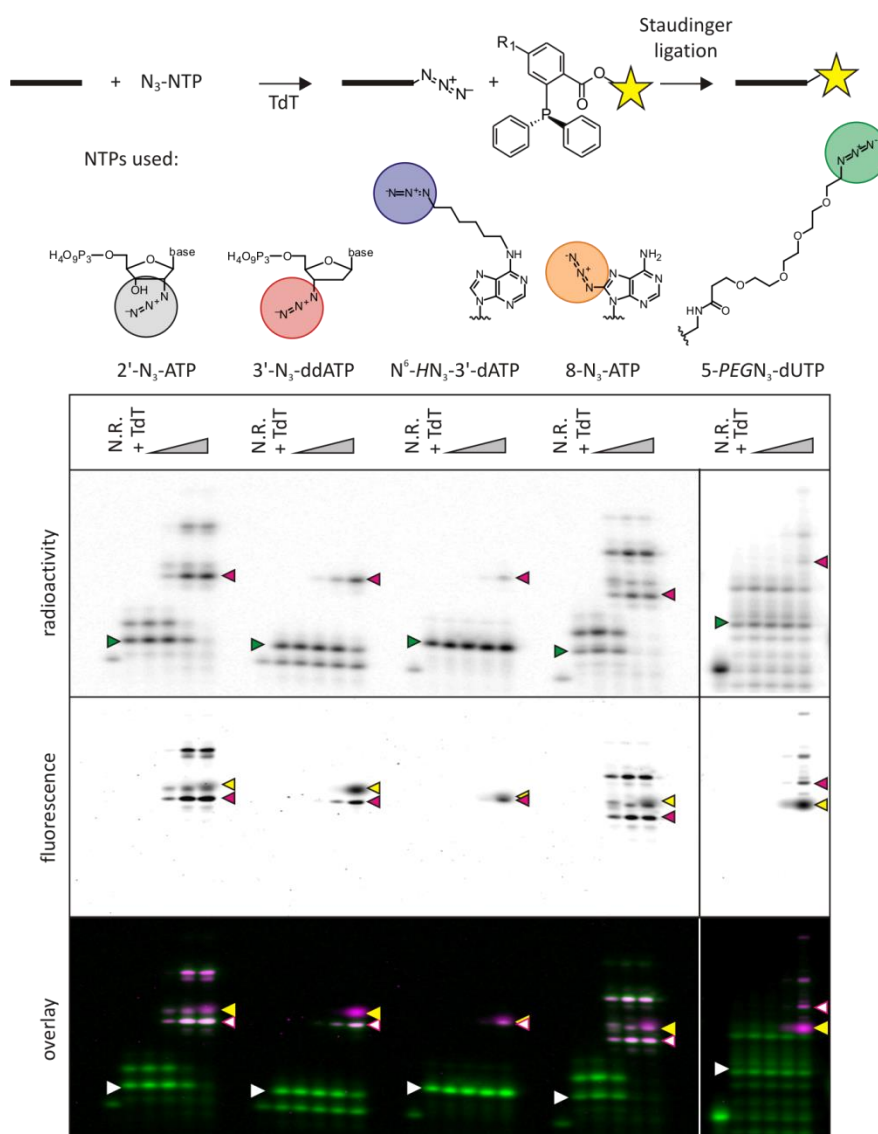
Supplementary Figure S4. CuAAC, SPAAC and DAR_{inv} at DNA 3'-termini. Click reactions between DNA2, TdT reacted with 5-*E*-UTP, 5-*O*-dUTP or N⁶-*P*-ATP (**A**), 5-*E*-dUTP (**B**), 5-*O*-dUTP or 5-DIBAC-dUTP (**C**) or with different Norb-dNTPs (**D**), as shown in Figure 4, with additional representation of fluorescent signals and overlay of radioactive and fluorescent signals. CuAAC after precipitation of TdT products (**A,B**); CuAAC or SPAAC (**C**) or DAR_{inv} (**D**) without purification of TdT products. In all cases, click reactions lead to clear band shifts and fluorescent bands appear at the shifted positions whenever a dye-labeled reaction partner was used. In (**D**) sequences carrying >2 modifications disappear after DAR_{inv}, while a strong DNA signal appears in the pocket. Because non-reacted DNA is still visible in equal amounts as after tailing reaction, we conclude that DNA bearing >2 Cy5 moieties attached by DAR_{inv} are too hydrophobic to enter gel pockets. Analysis by 15% sequencing PAGE. In overlays, radioactive signals are shown in green, fluorescent signals in magenta. Overlay of both leads to brighter or white color, and is used to verify product formation. N.R.: no reaction control/not reacted. Abbreviations: *E*: ethynyl, *O*: octynyl, Norb: norbornene, *P*: propargyl.



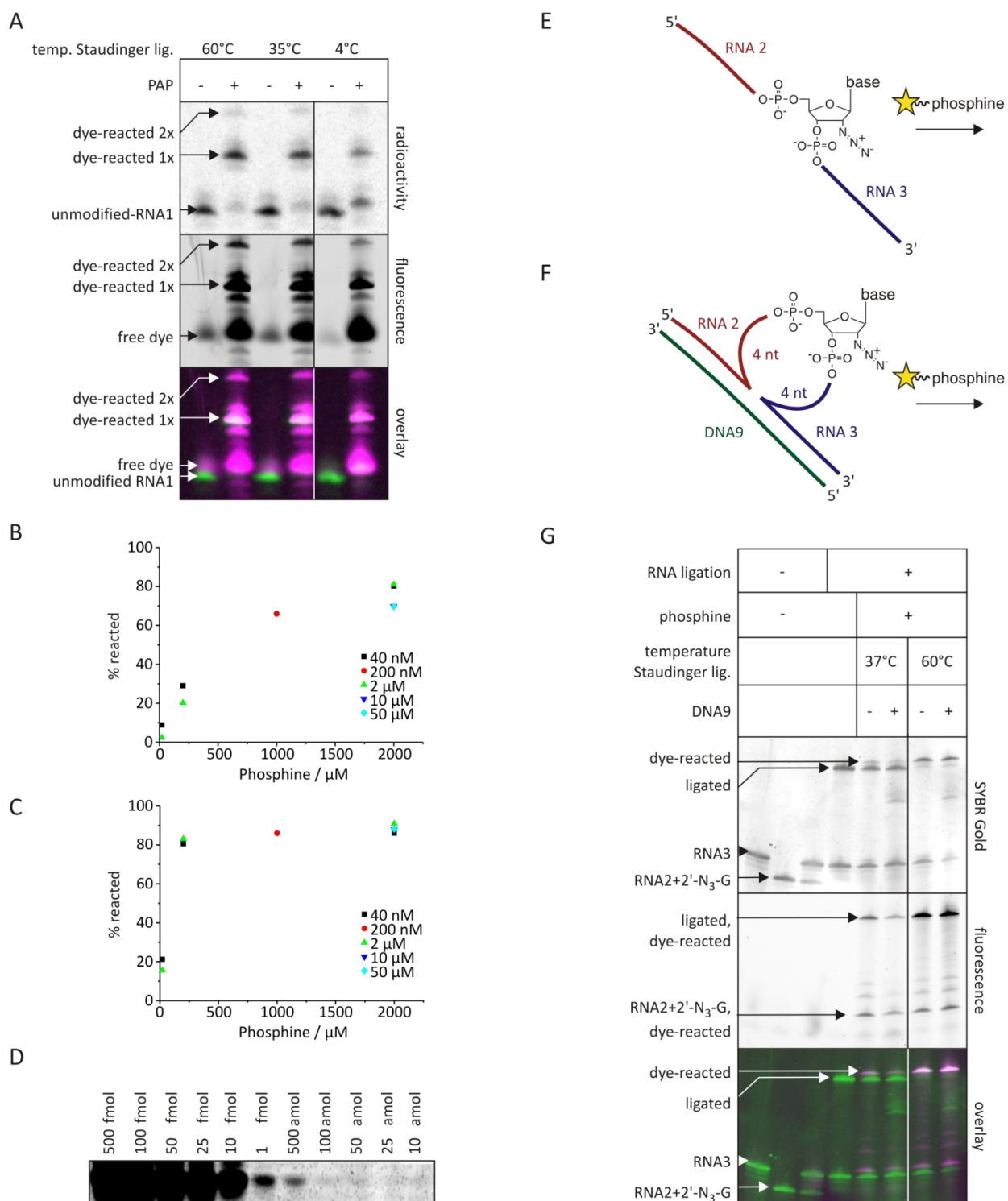
Supplementary Figure S5. CuAAC at the 3'-terminus of DNA2 tailed with different azido-modified NTPs to study influence of attachment position and linker. DNA (250 nM) was reacted with different concentrations (2.5 μ M, 25 μ M, 250 μ M or 2 mM) of Alexa Fluor 647 alkyne. Samples were analyzed by 15% seqPAGE. Product formation (of single-tailed/clicked DNA) was quantified ratiometrically making use of radioactive signals. Single tailed DNA is indicated by green triangles, single clicked DNA by triangles in magenta. The identity of click products was verified by the appearance of fluorescent bands that overlap the radioactive signal in the overlay, where radioactive signals are shown in green, and fluorescent signals in magenta.



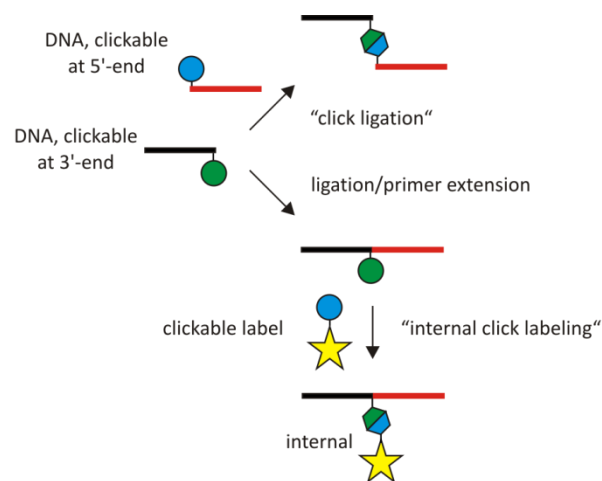
Supplementary Figure S6. SPAAC at the 3'-terminus of DNA2 tailed with different azido-modified NTPs to study influence of attachment position and linker. DNA (250 nM) was reacted with different concentrations (2.5 μ M, 25 μ M, 250 μ M or 2 mM) of DIBAC-Fluor-488. Samples were analyzed by 15% seqPAGE. Product formation (of single-tailed/clicked DNA) was quantified ratiometrically making use of radioactive signals. Single tailed DNA is indicated by green triangles, single clicked DNA by triangles in magenta. The identity of click products was verified by the appearance of fluorescent bands that overlap the radioactive signal in the overlay, where radioactive signals are shown in green, and fluorescent signals in magenta. Presence of both signals results in white or bright color. Double bands of click products (seen, e.g., for N^6 - HN_3 -3'-dATP) are due to different stereoisomers that sometimes exhibit slight differences in migration patterns.



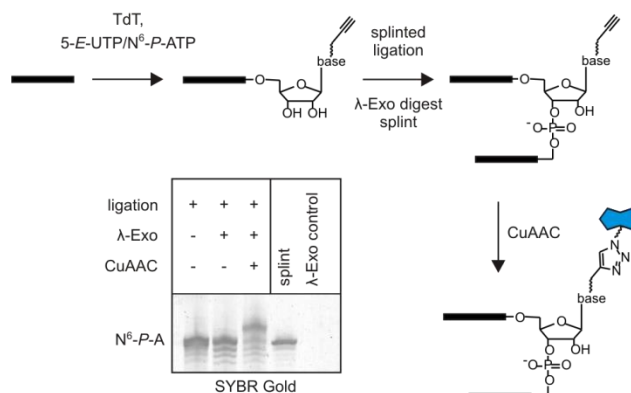
Supplementary Figure S7. Staudinger ligation at the 3'-terminus of DNA2 tailed with different azido-modified NTPs to study influence of attachment position and linker. DNA (250 nM) was reacted with different concentrations (2.5 μ M, 25 μ M, 250 μ M or 2 mM) of DyLight 488 phosphine. Samples were analyzed by 15% seqPAGE. Product formation (of single-tailed/clicked DNA) was quantified ratiometrically making use of radioactive signals. Single tailed DNA is indicated by green triangles, single clicked DNA by triangles in magenta. The identity of click products was verified by the appearance of fluorescent bands that overlap the radioactive signal in the overlay, where radioactive signals are shown in green, and fluorescent signals in magenta. Presence of both signals results in white or bright color. Signals caused by the dye which migrate close to the single-reacted bands, but do not correspond to radioactive signals and migrate in a comparable manner for all different DNAs are marked by yellow triangles. These signals are recognizable also by a "blurred" shape and can therefore be differentiated from labeled DNA. As a further confirmation of actual Staudinger ligation products, the shift caused by the attachment of the dye is equal for all different DNAs.



Supplementary Figure S8. Staudinger ligation on RNA. **(A)** Reaction of 2'-N₃-U containing RNA1 overnight with 2 mM DyLight 488 Phosphine at different temperatures, analyzed by 15% sequencing PAGE. **(B,C)** Reaction at 37°C with varying concentration of 2'-N₃-U-containing RNA1 and DyLight 488 Phosphine for 2 h **(B)** or overnight **(C)**. **(D)** Detection limit of Staudinger ligation product (37°C, 2 h, 2 mM DyLight 488 phosphine) in 12% sequencing PAGE. **(E,F)** Scheme of Staudinger ligation at internal position with or without helper DNA9. Modified from (9). **(G)** Staudinger ligation at an internal position. Analysis by 12% denaturing PAGE. Interestingly, unlike the situation in CuAAC (9), the use of helper DNA D9 does not increase the efficiency of Staudinger ligation at the internal position. In contrast, higher temperatures restore near-quantitative conversion of the internal azide. In **(A)** and **(G)**, lines indicate where different parts of the same gel have been combined for better comparison.



Supplementary Figure S9. Comparison of “click ligation” and “internal click labeling”. In “click ligation”, a new bond is formed between a 3'-modified and a 5'-modified DNA. The bond that is formed is non-natural. Modifications (e.g., azide and alkyne) are no longer available for further labeling after having reacted already in the “click ligation”. In our case, a 3'-modified DNA is enzymatically ligated or extended, to attach a second stretch of DNA. In this case, where a standard phosphodiester bond is formed between the two DNAs, the modification remains intact and can be used to attach a label of interest at an internal position of the resulting DNA. See also Supplementary Figure S10.



Supplementary Figure S10. Internal labeling of DNA using splinted ligation and CuAAC. Splinted ligation is performed first, followed by lambda-exonuclease (λ-Exo) treatment, which is used to remove the splint, and copper-catalyzed click reaction (CuAAC), in order to link biotin to the alkyne-labeled DNA. As a control, the splint alone is loaded without further treatment (splint) or after being subjected to lambda-exonuclease treatment under equal conditions (λ-Exo control). The control shows that the splint is completely digested under assay conditions. Furthermore, an excellent conversion of internally alkyne-labeled DNA can be observed in the CuAAC sample. 12% denaturing PAGE is shown after SYBR Gold staining.

Supplementary Table S1. Modified and non-modified nucleotides and click-reagents used in this study.

(Modified) NTPs (commercial) full name	Abbreviation	Provenience
2'-N ₃ -2'-dATP (Li-salt)	2'-N ₃ -ATP	tebu-bio
2'-N ₃ -2'-dCTP (Na-salt, Li-salt)	2'-N ₃ -CTP	IBA, tebu-bio
2'-N ₃ -2'-dGTP (Li-salt)	2'-N ₃ -GTP	tebu-bio
2'-N ₃ -2'-dUTP (Na-salt, Li-salt)	2'-N ₃ -UTP	IBA, tebu-bio
3'-N ₃ -3'-dATP (Na-salt)	3'-N ₃ -ATP	IBA
3'-N ₃ -2',3'-ddATP (Na-salt, Li-salt)	3'-N ₃ -ddATP	IBA, tebu-bio
3'-N ₃ -2',3'-ddCTP (Na-salt, Li-salt)	3'-N ₃ -ddCTP	IBA, tebu-bio
3'-N ₃ -2',3'-ddGTP (Li-salt)	3'-N ₃ -ddGTP	tebu-bio
3'-N ₃ -2',3'-ddTTP (Li-salt)	3'-N ₃ -ddTTP	tebu-bio
5-DBCO-dUTP (TEA-salt)	5-DIBAC-dUTP	Jena Bioscience
5-Ethynyl-dUTP (5-EdUTP, Na-salt)	5- <i>E</i> -dUTP	Jena Bioscience
5-Ethynyl-UTP (5-EUTP, Na-salt)	5- <i>E</i> -UTP	Jena Bioscience
8-N ₃ -ATP (Na-salt)	8-N ₃ -ATP	BIOLOG
Azide-PEG ₄ -aminoallyl-dUTP (TEA-salt)	5-PEGN ₃ -dUTP	Jena Bioscience
C8-Alkyne-dCTP (Na-salt)	5- <i>O</i> -dCTP	Base-click
C8-Alkyne-dUTP (Na-salt)	5- <i>O</i> -dUTP	Base-click
N ⁶ -(6-Azido)hexyl-3'-dATP (Na-salt)	N ⁶ -HN ₃ -3'-dATP	Jena Bioscience
N ⁶ -(6-Azido)hexyl-ATP (Na-salt)	N ⁶ -HN ₃ -ATP	Jena Bioscience
N ⁶ -(6-Azido)hexyl-dATP (Na-salt)	N ⁶ -HN ₃ -2'-dATP	Jena Bioscience
N ⁶ -Propargyl-ATP (Na-salt)	N ⁶ - <i>P</i> -ATP	Jena Bioscience
Norbornene-dATP (Na-salt)	7-Norb-dATP	Lab-synthesized (22)
Norbornene-dCTP (Na-salt)	5-Norb-dCTP	Lab-synthesized (22)
Norbornene-dUTP (Na-salt)	5-Norb-dUTP	Lab-synthesized (22)
Non-modified nucleotide(s) full name		Provenience
dATP, dCTP, dGTP, dTTP		Rapidozym / Steinbrenner
dUTP		USB
dITP		Jena Bioscience
ddATP, ddCTP, ddGTP, ddTTP		USB
NTP (ATP, CTP, GTP, UTP)		Sigma-Aldrich
ITP		Sigma-Aldrich
Click-Reagents (alternative commercial name in parentheses)		Provenience
Alexa Fluor 647 alkyne		Life Technologies
Alexa Fluor 647 azide		Life Technologies
Biotin-azide (amide)		synthesized by A. Samanta [5 in (10)]
Cy5 azide		Jena Bioscience
Cy5 tetrazine		gift from M. Wießler (DKFZ)
DIBAC Fluor 488 (sold as Dibenzylcyclooctyne Fluor 488)		Jena Bioscience
DyLight 488 phosphine		Thermo Scientific
THPTA		synthesized by A. Samanta (9)

Supplementary Table S2. List of oligonucleotides used in this study.

Oligonucleotide	Sequence	Provenience	Note
DNA1	5'-GATAATATGAAAGTGAGTTTC-3'	IBA/Biomers	
DNA2	5'-GGAGCTCAGCCTTCACTGC-3'	Biomers	
DNA2'	5'-GCAGTGAAGGCTGAGCTCC-3'	DNA2	Reverse complement of DNA2
DNA3	5'-NNNNNNNNNNNNNNNNNNNN-3'	Biomers	Randomized 21mer DNA
DNA4	5'-ATTGATGGTGCCTACAG-3'	IBA	
DNA4-6-FAM	5'-6-FAM-ATTGATGGTGCCTACAG-3'	Biomers	6-FAM labeled DNA4
DNA5	5'-GTGCGCTAGCAATATAGTTTAGCAGCAGTGAAGGCTGAGCTCC-3'	IDT	Splint/template for 5'-E-U-tailed DNA2
DNA6	5'-GTGCGCTAGCAATATAGTTTAGCAGCAGTGAAGGCTGAGCTCC-3'	IDT	Splint/template for N ⁶ -P-A-tailed DNA2
DNA7	5'-p-GCTAAACTATATTGCTAGCGCAC-3'	Biomers	Ligation fragment for ligation of DNA2
DNA8	5'-CGCCATTACTGGTTGCTGAAGCATCGTCGGGCCAC TGC GCGGTGAAGTCGATCCGCGGTAC-3'	IBA	Splint for ligation of RNA2 and RNA3 (9)
DNA9	5'-CGCCATTACTGGTTGCTGAAGCATCGTCGCGGTGAA GTCGATCCGCGGTAC-3'	IBA	Helper DNA for internal click reaction (9)
RNA1	5'-GCAAGCUGACCCUGAAGUUA-3'	Biomers	RNA to be tailed
RNA2	5'-GUGACCGCGGAUCGACUACCGCGCAGUG-3'	IBA	RNA to be tailed & ligated (9)
RNA3	5'-pGCCCGACGAUGCUUCAGCAACCAGUGUAAUGGCG-3'	Dharmacon	RNA ligation fragment (9)

Supplementary Table S3. List of scan settings for PAGE. (BP: band pass; SP: short pass).

Dye/phenomenon	Excitation	Emission
Alexa Fluor 488, Fluor 488, 6-FAM	488 nm	520nm BP 40
Alexa Fluor 647, Cy5	633 nm	670 nm BP 30
SYBR Gold - high sensitivity	488 nm	520 nm BP 40
SYBR Gold - low or medium sensitivity	532 nm	526 nm SP